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Agmatine Is Synthesized by a Mitochondrial Arginine Decarboxylase in Rat Brain

GEN LI, SOUNDARARAJAN REGUNATHAN, AND
DONALD J. REIS

Division of Neurobiology
Department of Neurology and Neuroscience
Cornell University Medical College
New York, New York 10021

We recently discovered that agmatine (decarboxylated arginine) is contained in bovine brain.[1] Moreover, the amine, never before detected in mammals,[2] has properties of a clonidine-displacing substance (CDS); it binds with reasonable affinities (K_i: 1-4 μM) to α_2-adrenergic receptors and to all classes of imidazoline (I-) receptors.[1] In bacteria, fungi, some parasites, and marine animals,[2–5] agmatine is synthesized from arginine by a soluble enzyme, arginine decarboxylase (ADC). Our finding that ADC was expressed in rat brain[1] was the first demonstration of the enzyme in mammals. Moreover, it indicated that mammalian agmatine is a product of local biosynthesis and not attributable to dietary or enteric bacterial sources.

In *Escherichia coli* there are two soluble cytosolic isoforms: one is constitutive or biosynthetic,[6] the other inducible or biodegradative.[2] A third form of ADC which is membrane associated has been found in nematode *C. elegans.*[7] We sought to determine if mammalian brain ADC was similar to any of these other forms.

MATERIALS AND METHODS

Rat brains were homogenized in HEPES-sucrose buffer (5 mM HEPES, pH 7.4; 2 mM DTT; 0.5 mM PMSF; 0.2 mM EDTA) with a Teflon-glass homogenizer and centrifuged at 1,000 × *g* for 10 minutes. The pellet (P1) containing the nuclear fraction was saved for enzyme assays. The supernatant was centrifuged at 12,000 × *g* to produce a crude mitochondrial/synaptosomal pellet (P2). The resulting supernatant was centrifuged at 30,000 × *g* for 20 minutes generating a pellet (P3) containing the plasma/microsomal membrane fraction. This was used for enzyme assays. Separate mitochondrial or synaptosomal fractions were isolated from the P2 pellet by Percoll density gradients.[8] Membranes from each fraction were used for enzyme assays.

Except for studies on enzyme distribution in each subcellular compartment, ADC activity was measured in the P2 pellet which contained mitochondrial/synaptosomal membranes. Arginine decarboxylase was assayed by the method of Wu and Morris[9] measuring conversion of ^{14}C-arginine to $^{14}CO_2$. In brief, the tissue pellet was suspended in pre-chilled subcellular fractionation buffer without sucrose, homogenized by a Polytron (Brinkman, setting 6 for 2 × 15 s), and centrifuged at 100,000 × *g* for

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15 minutes. The enzyme reaction was performed in glass tubes with a center well inserted into a tightly closed rubber stopper. The center wells contain strips of filter paper moistened with benzethionium or methylbenzethionium hydroxide to trap the $^{14}CO_2$ produced. The reaction mixture consisted of 500 μl of assay buffer: 10 mM Tris-HCl, pH 8.25, at 30°C; 1 mM DTT; 0.5 mM PMSF; 0.2 mM EDTA; 1 mM $MgSO_4$; 0.2 mM L-arginine and 0.4 μCi of L-[1-^{14}C]-arginine, specific activity 55 mCi/mmol. It was incubated at 30°C for 1 hour in a shaking water bath and the reaction terminated by the addition of 100 μl of 40% trichloroacetic acid injected through the rubber stopper. After further incubation for 20 minutes at 37°C, the filter paper strips were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Verification of the product was obtained independently by demonstrating generation of [^{3}H]agmatine from [^{3}H]arginine by HPLC (data not shown).

Ornithine decarboxylase activity was measured by modification of a similar CO_2-trapping method[7] using 0.2 mM L-ornithine and L-[1-^{14}C]-ornithine (specific activity 55 mCi/mmol) as substrates. Protein was measured with a commercial protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

RESULTS

Stability. Arginine decarboxylase is labile. Hence, all enzyme activity was lost within a few hours in whole brain when stored on ice, whereas about 50% of activity was lost overnight when stored at 4°C as a membrane suspension. The enzyme was sensitive to detergents. At concentrations of 1%, Triton X-100, NP 40, or CHAPS totally abolished ADC activity, and even at 0.1% concentration, enzyme activity was substantially decreased.

Subcellular Distribution. The subcellular distribution of enzyme was measured after separation into nuclear, mitochondrial/synaptosomal, and plasma membrane fractions. The highest activity, 9.69 nmol CO_2/h/mg protein, or ~89% of total activity was restricted to the mitochondrial/synaptosomal membrane fraction. The activity of the plasma membrane (0.77 nmol. CO_2/h/mg protein) and the nuclear pellet (<0.48 nmol CO_2/h/mg protein) was ~7% and ~4% of total activity, respectively. After further fractionation of the mitochondrial/synaptosomal fraction 80% of total activity (88.67 nmol CO_2/h/mg protein) appeared in mitochondrial membranes. Much of the remaining 20% of ADC activity within the synaptosomal membrane (20.22 nmol CO_2/h/mg protein) was probably attributable to mitochondria, because these organelles are abundant in synaptosomes. Thus, ADC appears to be highly, if not exclusively, associated with mitochondrial membrane.

Kinetics. The activity of ADC was linear with respect to reaction time to 90 minutes and with protein concentrations from 0.5-40 mg/ml. Under optimal reaction conditions, the K_m for arginine, as determined by double-reciprocal (Lineweaver-Burk) plots, was 0.75 mM. The V_{max} was 2.22 nmol/h/mg protein.

pH and Temperature Optima. The optimal pH for brain ADC activity was 8.25, close to that of the biosynthetic ADC of *E. coli.*[10] By contrast to other forms of ADC, the temperature optimum was 30°C. At 37°C, the enzyme was only about one third as active as at 30°C.

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TABLE 1. Effect of Substrate Analogs and Polyamines on Arginine Decarboxylase Activity[a]

Compound	Relative Activity (%)
None	100
Amino acids:	
L-Ornithine	18
L-Lysine	59.8
L-Glutamine	79.4
L-Histidine	93.7
D-Arginine	94.7
D-Ornithine	92.3
DFMO	98.5
Polyamines:	
Spermine	18.8
Spermidine	29.4
Agmatine	66.5
Putrescine	66.6
Nitric oxide synthase inhibitors:	
N^G-nitroarginine	79.6
N^G-methylarginine	78.8

[a] The activity of ADC was measured in rat brain mitochondrial membrane for 1 hour at 37°C. Control activity was 88.6 nmol CO_2/h/mg protein. All compounds were tested at 1 mM against the substrate concentration of 0.2 mM arginine.

Inhibition by Substrate Analogs. To determine the effect of substrate analogs on ADC activity, several amino acids were tested for their ability to inhibit the enzyme. As shown in TABLE 1, although ornithine inhibited ADC activity by 82% at 1 mM concentration, lysine inhibited the enzyme by 40%. Therefore, rat brain ADC has properties similar to those of ADC of *C. elegans*[7] but not of *E. coli* and can be considered arginine/ornithine decarboxylase. However, difluoromethylornithine (DFMO), a universal and irreversible inhibitor for all forms of ornithine decarboxylase,[11] could not inhibit rat brain ADC, indicating the distinction between the two forms of the enzyme.

Because in bacteria agmatine is a precursor of polyamines,[12] the effect of several polyamines on ADC activity was examined. Arginine decarboxylase activity was inhibited, in rank order by: spermine > spermidine > agmatine > putrescine. At a concentration of 1 mM, which was fivefold higher than the substrate concentration (TABLE 1), spermidine and spermine blocked 81% and 70% activity, whereas agmatine and putrescine blocked only one third of the enzyme activity. Arginine decarboxylase was not inhibited by the nitric oxide synthase inhibitors N^G-nitroarginine and N^G-methylarginine.

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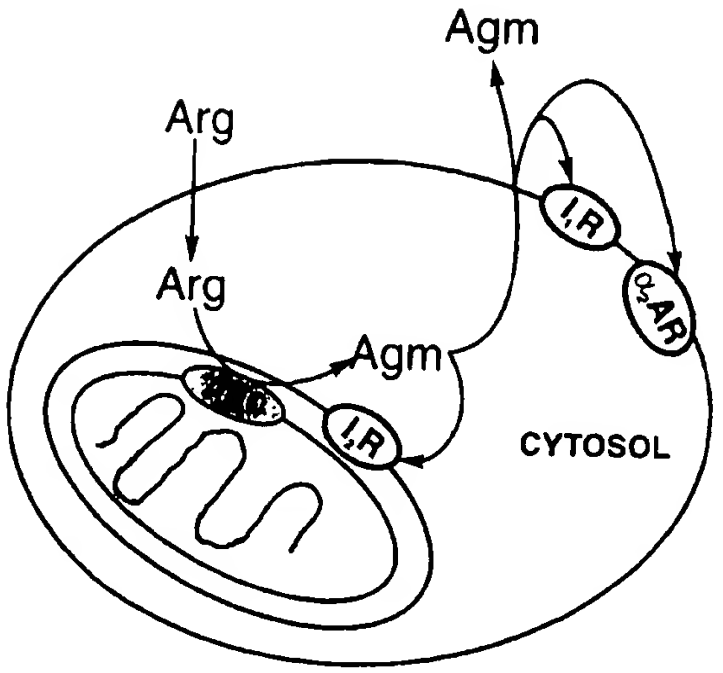

FIGURE 1. Schematic outline of the intracellular biosynthetic pathway for agmatine and its relationship to imidazoline (I-) and α_2-adrenergic receptors. See text for details.

DISCUSSION

This study confirms our previous observation that the rat brain expresses an enzyme that can synthesize agmatine and CO_2 from L-arginine and hence by definition represents an ADC.[1] The enzyme differs from the soluble bacterial forms in that it is associated with mitochondrial membranes. Like the constitutive form of *E. coli*,[10] it is optimally active at a high pH (8.25), but unlike that enzyme it does not require Mg^{2+} (unpublished observations).

Because rat brain ADC also uses ornithine as a substrate (K_m: 0.25 mM), it can be considered an arginine/ornithine decarboxylase. It differs, however, from ODC, a soluble cytoplasmic enzyme, which is irreversibly inhibited by DFMO. By contrast, DFMO is inactive against rat brain ADC. Rat brain ADC is most similar to a membrane-associated ODC isolated from the nematode C. elegans[7] which also uses arginine and ornithine as substrates; however, it is not known if the nematode enzyme is mitochondrial. However, the two enzymes also differ with respect to K_m, pH, and temperature optima and by the fact that the nematode enzyme is inhibited by DFMO.[7] Thus, rat brain ADC appears unique.

The localization of ADC to mitochondrial membranes is of special interest in view of the fact that imidazoline receptors of the I_2 subclass are also localized there.[13,14] FIGURE 1 schematically indicates the relation between the substrate, L-arginine, ADC, agmatine, and I- and α_2-adrenergic receptors in a typical cell. It emphasizes that the substrate L-arginine, which enters the cell by facilitated transport, is converted to agmatine on the mitochondrial membrane. Agmatine, so synthesized, can either bind to I_2 receptors on the mitochondrion, to α_2-adrenergic and possibly I_1 receptors on plasma membranes, or it can be released extracellularly, as evidenced by the presence of agmatine in serum.[14] Within the cell, agmatine may also regulate its own synthesis by feedback inhibition of ADC.

The data therefore suggests that ADC is a component of a complex and highly regulated system within cells generating agmatine, an amine that may act within and

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beyond the cell as an autocrine, paracrine, and hormonal messenger. We have argued[16] that agmatine may also be a novel neurotransmitter. If so, ADC undoubtedly participates in a potentially new neurotransmitter/neuromodulator network in the CNS.

CONCLUSIONS

1. Rat brain expresses ADC which differs from plant and bacterial forms by its localization to mitochondrial membranes and utilization of ornithine as well as arginine as substrate; yet, it is not a typical ODC as it is not cytosolic or inhibited by DFMO.

2. The colocalization of I_2 receptors and ADC to mitochondria suggests that agmatine may be an important intracellular message possibly regulating, through receptor mediation, its own biosynthesis. The presence of ADC and agmatine in mammalian brain raises questions of whether it might be an alternative pathway for polyamine biosynthesis in mammalian tissue.

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